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Double compartmentalization is further confirmed by quantitative anomaly analysis

To further confirm double compartmentalization of the NRK cell membrane, we performed another quantitative analysis using the MSD (see the Materials and methods), i.e., the mean log(MSD/time) for gold-tagged DOPE is plotted as a function of log(time) based on the data obtained at time resolutions of 25 μs, 0.22 ms, 2 ms, and 33 ms (Fig. S1). The slope in this display is sensitive to anomaly of diffusion: due to the relationship of log(MSD/time) = (α – 1) log(time) ($0 \leq \alpha \leq 1$), α parametrizes the level of anomaly (Saxton, M.J. 1994. *Biophys. J.* 66:394–401; Saxton, M.J. 1996. *Biophys. J.* 70:1250–1262; Feder, T.J., I. Brust-Mascher, J.P. Slattery, B. Baird, and W.W. Webb. 1996. *Biophys. J.* 70:2767–2773). In the case of simple Brownian diffusion, the plot becomes flat ($\alpha - 1 = 0$) and $\alpha = 1$. When the diffusion is anomalous, α becomes <1, giving a negative slope ($\alpha - 1 < 0$) to the plot. For DOPE molecules undergoing free Brownian diffusion in LUVs (Fig. S1), the plot is almost flat between 100 μs and 10 ms when observed at a 25-μs resolution. The best fit for the plot yields an α of 0.97 (∼1), which indicates that DOPE in LUVs is undergoing simple Brownian diffusion. On the other hand, the plot for the cell membrane exhibits multiple negative slopes depending on the observation time intervals. The plot can be fitted with three lines with αs of 0.74 (<12 ms), 0.55 (12–590 ms), and 0.79 (>590 ms). The transitions were found to occur at 12 ms and 0.59 s, comparable to the residency times within 230-nm (11 ms) and 750-nm (0.65 s) compartments for DOPE hop diffusion, respectively. Therefore, we interpret this time dependence as a result of (a) DOPE being corralled inside 230-nm compartments for an average of 11 ms while undergoing free diffusion inside the compartment, (b) DOPE being corralled inside 750-nm compartments for an average of 0.65 s while undergoing hop movement among 230-nm compartments (because we are using the data for gold-tagged DOPE, the hop rate among 750-nm compartments is 0.65 s rather than the 0.33 s estimated for Cy3–DOPE), and (c) DOPE undergoing hop movement among 750-nm compartments once every 0.65 s. Although anomalous diffusion in the cell membrane could be caused by various kinds of obstacles and binding sites (Saxton, 1994, 1996), the good agreement of these transition times in Fig. S1 with the residency times (11 ms and 0.65 s) supports the predominant involvement of double compartmentalization (into 230- and 750-nm compartments) in limiting the macroscopic diffusion rate of DOPE.

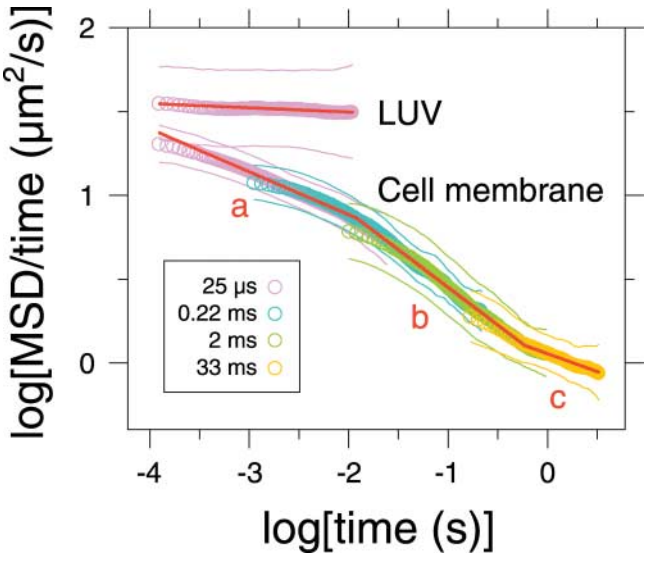

Figure S1. **Plots of log(MSD/time) against log(time) provide useful information on the changes of diffusion characteristics that depend on the observation time intervals.** MSD of the trajectories was estimated using data obtained at the time resolutions of 25 μs (5,000 frames; purple), 0.22 ms (5,000 frames; blue), 2 ms (2,500 frames; green), and 33 ms (500 frames; orange) for the time windows where theoretically expected statistical errors in MSD are <40% (Qian et al., 1991). Then the mean log(MSD/time) values (circles) and their standard deviations (curves) for each time resolution were plotted as a function of log(time). The shorter (log(time) < −1.5) and the longer (−1.5 < log(time)) periods contain 1,113 and 1,388 time points, respectively, which would give rather uniform weight over the entire time window when least square fitting is performed for these points as described below. Normal (simple Brownian) and anomalous diffusion can be distinguished in this display as having no time dependence (slope ∼ 0) and having negative slopes, respectively (Saxton, 1994, 1996; Feder et al., 1996). The best fit on the data obtained for the cell membrane using the continuous three linear segments (red solid line, representing three anomalous exponents) gives transitions at 12 ms and 0.59 s. These three segments are consistent with (a) the confinement of freely diffusing DOPE within a 230-nm compartment for an average of 11 ms, (b) the confinement of DOPE undergoing consecutive hops over 230-nm compartments within a 750-nm compartment for an average of 0.65 s, and (c) consecutive hop movement of DOPE over many 750-nm compartments. The slope in the experimental data is shallower at the high time resolution end (left end) because at these time intervals, DOPE molecules that are not located near the compartment boundaries are undergoing simple Brownian diffusion. In addition, if we had employed lower resolution or longer time intervals of observation, the slope at the right end would have become shallower because DOPE molecules would have looked as if they were undergoing simple diffusion by hopping from one (750 nm) compartment to another. For comparison, the plot for the trajectories in LUVs (2,250 frames; $n = 17$) and the best regression result (red line, $\alpha = 0.97$) are also shown.